



Li, H., Pesce, E., Sheppard, D. N., Singh, A. K., & Pedemonte, N. (2018). Therapeutic approaches to CFTR dysfunction: from discovery to drug development. *Journal of Cystic Fibrosis*, 17(2), S14-S21. <https://doi.org/10.1016/j.jcf.2017.08.013>

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THERAPEUTIC APPROACHES TO CFTR DYSFUNCTION: FROM DISCOVERY TO DRUG DEVELOPMENT

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Keywords:

CFTR Cl⁻ channel; F508del-CFTR; CFTR correctors; pharmacological chaperones; proteostasis regulators, artificial anion transporters (anionophores)

ABSTRACT:

Cystic fibrosis (CF) mutations have complex effects on the cystic fibrosis transmembrane conductance regulator (CFTR) protein. They disrupt its processing to and stability at the plasma membrane and function as an ATP-gated Cl⁻ channel. Here, we review therapeutic strategies to overcome defective CFTR processing and stability. Because CF mutations have multiple impacts on the assembly of CFTR protein, combination therapy with several pharmacological chaperones is likely to be required to rescue mutant CFTR expression at the plasma membrane. Alternatively, proteostasis regulators, proteins which regulate the synthesis, intracellular transport and membrane stability of CFTR might be targeted to enhance the plasma membrane expression of mutant CFTR. Finally, we consider an innovative approach to bypass CFTR dysfunction in CF, the delivery of artificial anion transporters to CF epithelia to shuttle Cl⁻ across the apical membrane. The identification of therapies or combinations of therapies, which rescue all CF mutations, is now a priority.

HIGHLIGHTS:

- Many CF mutations disrupt the processing and/or function of the CFTR Cl⁻ channel
- Combinations of CFTR correctors are needed to optimally rescue CF processing mutants
- Proteostasis regulators have been identified that rescue F508del-CFTR
- Artificial anion transporters are an alternative strategy to bypass CFTR loss in CF

Cystic Fibrosis (CF) is caused by loss-of-function mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR) [1], an ATP-binding cassette (ABC) transporter expressed at the apical membrane of many epithelia, which functions as an ATP-gated anion channel [2]. Approximately 2,000 mutations have been identified in the *CFTR* gene (<http://www.genet.sickkids.on.ca/app>), but most are very rare and not all lead to a defective protein or manifest as a clinical phenotype [3].

The most common CF mutation is the deletion of phenylalanine 508 (F508del), which reduces the intrinsic stability of the first nucleotide-binding domain (NBD1) and perturbs interactions between NBD1 and NBD2 as well as those between NBD1 and the membrane-spanning domains (MSDs) [4]. Such abnormalities lead to misfolding of F508del-CFTR, ubiquitination in the endoplasmic reticulum (ER) and premature degradation by the proteasome [5,6]. Any F508del-CFTR that reaches the plasma membrane exhibits reduced stability [7] and defective channel gating [8].

To address the most prevalent defects leading to CF, two biomolecular activities are required, namely CFTR correctors to increase the amount of properly folded F508del-CFTR protein at the plasma membrane, and CFTR potentiators to allow effective gating (i.e. function) of F508del-CFTR [4,9]. When combined, these biomolecular activities restore transepithelial Cl⁻ transport to CF airway epithelia expressing F508del-CFTR, improving hydration and restoring mucociliary clearance [10]. At present, two orally-bioavailable small molecules with biomolecular activities targeting mutant CFTR are commercially available for CF patients: the CFTR corrector Lumacaftor (VX-809) and the CFTR potentiator Ivacaftor (VX-770) [10,11]. Ivacaftor is approved for use with 33 CF mutations causing gating defects (<https://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm559212.htm>). By contrast, Lumacaftor-Ivacaftor combination therapy (Orkambi) is currently available only to CF patients homozygous for F508del-CFTR [12].

CFTR potentiators bind directly to mutant CFTR to improve channel gating after CFTR phosphorylation by PKA [9]. By contrast, some CFTR correctors

act as pharmacological chaperones while others are proteostasis regulators [13,14]. Pharmacological chaperones act directly on mutant CFTR, stabilizing specific CFTR domains and/or improving the interactions of different CFTR domains [15]. Proteostasis regulators modulate the proteostasis environment, leading to beneficial effects on CFTR processing and plasma membrane stability [14]. Regardless of their mechanism of action, the use of a single CFTR corrector is insufficient to achieve therapeutically-relevant rescue of F508del-CFTR [15,16]. The data suggest that drug therapy for CF will require combinations of CFTR correctors exploiting different mechanisms of action with additive/synergistic efficacies [17,18].

Other therapeutic strategies for CF are termed mutation-independent because they are applicable to all individuals with CF irrespective of the CF mutations that they carry. One mutation-independent therapeutic strategy is to bypass CFTR dysfunction by activating other apical membrane Cl⁻ channels in CF epithelia [19]. An innovative approach to bypass CFTR dysfunction is to deliver to the apical membrane of CF epithelia artificial anion channels or transporters [20]. Here, we review selectively recent progress in the development of pharmacological chaperones, proteostasis regulators and artificial anion transporters.

PHARMACOLOGICAL CHAPERONES TO RESTORE CFTR FUNCTION

The biggest recent breakthrough in CF therapeutics has been the development of CFTR potentiators and CFTR correctors that target directly the basic defect in CF, mutant CFTR. Due to the significant progress made towards understanding the relationship between CF genotype and subsequent causes of phenotype variation in CF, different *CFTR* variants can be classified as “theratypes”, depending upon their response to these CFTR modulators [3]. Indeed, these small molecule modulators most likely serve as personalized medicines for CF, which work by disease-modifying mechanisms [18].

Early studies of CFTR correctors identified that there was a “therapeutic ceiling” with maximal correction about 20% wild-type CFTR function [21,22]. An explanation for the limited effectiveness of first generation CFTR correctors is the wide-ranging impact of F508del-CFTR on CFTR assembly, perturbing domain-domain interactions [4]. Studies of revertant mutations demonstrated that the therapeutic ceiling could be exceeded by simultaneously targeting multiple defects in CFTR folding and assembly [15,16]. These data provided the rationale for combination therapy with CFTR correctors, pharmacological chaperones that bind directly to mutant CFTR to exert their effects [23,24]. Figure 1 provides examples of different CFTR correctors (C1- and C2-type) in development by pharmaceutical companies. C1-type correctors target early folding of mutant CFTR while C2-type correctors have novel mechanisms of action that complement C1-type correctors. When used together, either by themselves or with CFTR

amplifiers (small molecules which target an early step in the biosynthesis of CFTR to increase the amount of CFTR protein [25]), C1- and C2-type correctors are expected to deliver sufficient F508del-CFTR to the plasma membrane to achieve far greater clinical benefit than that observed with Lumacaftor alone [12]. When used together with a CFTR potentiator (Figure 1), triple combination therapy is expected to be transformative for individuals with the F508del-CFTR mutation.

CFTR Modulators

Modulators targeting the F508delCFTR maturation and other mutations

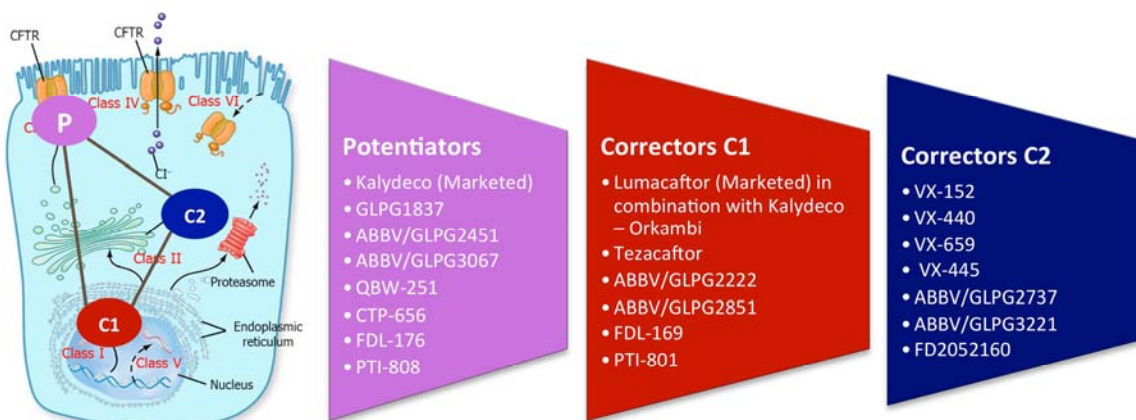


Figure 1: Triple combination therapy for CF. The molecular mechanisms of CFTR dysfunction in CF are shown together with small molecule CFTR modulators that act as potentiators (P), C1-type correctors and C2-type correctors. Marketed drugs and drug candidates in development for the three classes of CFTR modulators are listed. Pharmaceutical companies involved with the development of these drugs are AbbVie/Galapagos, Concert Pharmaceuticals, Flatley Discovery Lab, Novartis, Proteostasis Therapeutics and Vertex Pharmaceuticals.

One of the major challenges being faced by future CFTR correctors is the need for broader beneficial efficacy for all CF patients (i.e. targeting all CF mutations disrupting CFTR processing and membrane stability). Just as for F508del-CFTR, triple combination therapy consisting of two corrector molecules and a potentiator will likely have greatest benefit for rare CF mutations (Figure 1). Of all the different combinations that are currently in development, ones that achieve safe and efficacious “best-in-class” profiles, benefiting all CF patients will prevail in the end.

PROTEOSTASIS REGULATORS

Several proteins have already been identified that could represent useful drug targets for a CF therapy based on proteostasis modulation [13,14,26]. Molecular chaperones such as Hsp90 and its co-chaperone partner AHA1 are thought to play a key role in facilitating CFTR folding [27], as well as targeting folding-deficient F508del-CFTR for degradation (Figure 2). Thus, pharmacological inhibition of the Hsp90-AHA1 chaperone complex appears beneficial to prevent proteolysis of mutant CFTR and rescue its residual activity. Consistent with this idea, Ihrig and Obermann [28] identified two molecules that restored Cl⁻ channel activity to F508del-CFTR by inhibiting the Hsp90-AHA1 chaperone complex. Interestingly, the Hsp90-AHA1 inhibitors were most effective in combination with the CFTR corrector Lumacaftor and might therefore serve as leads that can be further developed into a combination therapy to treat CF patients [28].

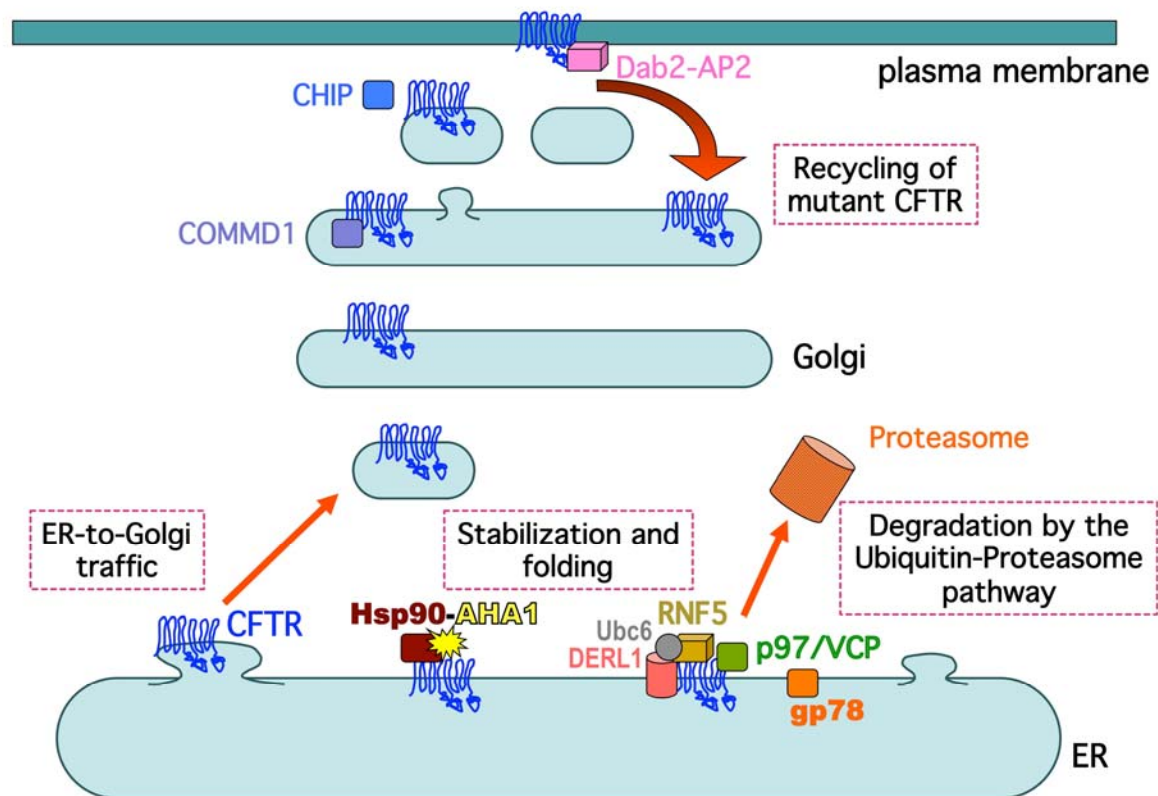


Figure 2: Proteostasis regulators as targets for CF drug development. The schematic shows the synthesis of CFTR in the ER and its transport to and recycling from the plasma membrane. Selected proteostasis regulators that represent targets for CF drug development are indicated and discussed in the text. Abbreviations: AHA1: Activator of Hsp90 ATPase Activity 1; AP2: Assembly Polypeptide-2; CHIP: Hsp70-interacting protein; COMMD1: Copper Metabolism Domain Containing 1; Dab2: Disabled-2; DERL1: Derlin-1; p97/VCP: 97 kDa

Valosin Containing Protein; RNF5: Ring Finger Protein 5; Ubc6: ubiquitin-conjugating enzyme E2 6.

Other possible drug targets are proteins in the ER quality control pathway known as ER-associated degradation (ERAD), including chaperones, components of the retro-translocon (DERL1, p97/VCP) [29], proteins responsible for extracting ubiquitinated substrates from the ER membrane (gp78) [30] and cytosolic and membrane-associated E3 ubiquitin ligases (e.g. RNF5). RNF5 (also known as RMA1) is a component of an ER-associated E3 ubiquitin ligase complex that contains Ubc6e and the transmembrane quality-control factor DERL1 [29] (Figure 2). The RNF5 E3 complex appears to sense the assembly status of N-terminal regions of CFTR at a folding step that occurs prior to NBD2 synthesis and is therefore defective in F508del-CFTR [31].

Tomati et al. (2015) demonstrated that RNF5 is a potential CF drug target using cultured cells and transgenic mice. *In vitro* studies showed that RNF5 knockdown strongly enhanced F508del-CFTR activity and increased the traffic of immature, partially glycosylated CFTR to the plasma membrane by an unconventional secretory pathway dependent on GRASP protein activity [32]. Rescue of mutant CFTR by RNF5 knockdown also displayed additive/synergistic effects with the CFTR corrector Lumacaftor [32], suggesting that each manipulation affects distinct pools of mutant CFTR.

To investigate whether RNF5 suppression might rescue F508del-CFTR *in vivo*, transgenic F508del-CFTR mice were crossed with RNF5 knockout mice [32]. Although homozygous F508del-CFTR mice lack the respiratory symptoms characteristic of CF in humans, they display CF gastrointestinal pathology [33]. Of note, *in vivo* RNF5 suppression ameliorated the gastrointestinal pathology observed in homozygous F508del-CFTR mice [32]. The number of animals with severely reduced body weight was significantly reduced, as well as fecal loss of biliary acids [32]. In parallel, short-circuit current measurements of duodenal Cl⁻ secretion in RNF5 knockout, homozygous F508del-CFTR mice showed a 5-fold increase in CFTR activity [32]. Together the *in vitro* and *in vivo* data demonstrate clearly the benefits of RNF5 knockdown [32]. Thus, RNF5-targeting inhibitors have the potential to ameliorate disease in CF patients bearing the F508del mutation.

Other proteins that constitute putative drug targets for CF are COMMD1 and CHIP (Figure 2). Indeed, COMMD1-mediated ubiquitylation regulates protein trafficking to the plasma membrane, and it acts either by enhancing the cell surface expression of CFTR or the internalization of the epithelial Na⁺ channel (ENaC) [26,34]. The ubiquitin E3 ligase CHIP participates in two different steps of CFTR biogenesis and processing [26]. In particular, CHIP, in complex with the cytosolic Hsc/Hsp70, cooperates with the ER membrane-associated ubiquitin ligases complex (containing RNF5, Ubc6e,

and DERL1) to triage wild-type and F508del-CFTR. Interestingly, CHIP also plays a role at the plasma membrane, where it is responsible for ubiquitylation of the mutant channel, acting in concert with Dab2 to target rescued F508del-CFTR to the lysosome [26].

Luciani et al. [35] demonstrated that the accumulation of misfolded F508del-CFTR in protein aggregates is a consequence of defective autophagy caused by transglutaminase-mediated depletion of beclin-1. Building on these data, De Stefano et al. [36] showed that restoration of beclin-1-dependent autophagy using the FDA-approved drug cysteamine reduces the mortality of CF mice bearing the F508del mutation and improves F508del-CFTR protein expression in the nasal epithelium of CF patients homozygous for F508del-CFTR. These data encourage further clinical testing of cysteamine in CF patients.

Once delivered to the plasma membrane, wild-type CFTR resides for prolonged periods (half-life, ~24 h), whereas F508del-CFTR is highly unstable (half-life, ~4 h) [7]. The reduced stability of F508del-CFTR at the plasma membrane reflects both the intrinsic instability of the mutant protein itself [37,38] and accelerated endocytosis of the mutant protein [39,40]. Therefore, addressing the plasma membrane instability of rescued F508del-CFTR might represent a therapeutic approach complementary to biosynthetic rescue of the mutant protein. Targets to improve CFTR stabilization at the apical plasma membrane include proteins involved in either endocytic retrieval or lysosomal degradation of the protein. The Dab2-AP2 complex facilitates endocytosis of CFTR by recruiting it with adaptor proteins (Figure 2). Consistent with this idea, RNAi-induced depletion of Dab2 inhibited F508del-CFTR endocytosis, increasing the half-life of the mutant CFTR by 91% [41]. Moreover, a peptide that binds the Dab2 DH peptide-binding pocket regulates CFTR abundance at the plasma membrane [42]. Therefore, peptides or drug-like small molecules, which inhibit the Dab2 DH peptide-binding pocket, might provide pharmacological approaches to stabilise F508del-CFTR at the plasma membrane.

ARTIFICIAL ANION TRANSPORTERS

Some CF mutations currently appear less amendable to rescue with CFTR modulators. For these CF mutations, mutation-independent therapies are the therapeutic strategy of choice. One approach to mutation-independent therapy aims to restore transepithelial Cl⁻ secretion to CF epithelia by utilising other apical membrane pathways for anion movement. Most work in this area has focused on endogenously expressed apical membrane Cl⁻ channels, particularly the Ca²⁺-activated Cl⁻ channel TMEM16A [19]. However, the Cl⁻ channel SLC26A9 has emerged as an attractive target because it mediates sustained Cl⁻ secretion, whereas that of TMEM16A is transient [19].

An innovative alternative approach to bypass CFTR dysfunction is to deliver to the apical membrane of CF epithelia an exogenous pathway for transmembrane anion movement. The absence of suitable natural products has stimulated efforts to develop artificial anion channels and transporters. Proof of principle for this approach was achieved using peptides based on the Cl⁻-selective pore of the glycine receptor [43].

Other studies have sought to develop artificial anion transporters (anionophores) that shuttle Cl⁻ across a lipid bilayer by a mechanism similar to that of the K⁺ ionophore valinomycin, which acts as a mobile carrier [20,44] (Figure 3). Using the steroid cholic acid as a scaffold, the Anthony P. Davis group generated a family of anionophores termed cholapods [44]. These anionophores possess pre-organised anion-binding sites capable of very high affinities linked to lipophilic scaffolds, which promote solubility in lipid bilayers [45,46]. Using synthetic phospholipid vesicles, cholapods were shown to transport Cl⁻ across lipid bilayers with high affinity (i.e. cholapod:lipid ratios of 1:250,000) by an anion exchange mechanism and with renal epithelia, they were demonstrated to generate Cl⁻-selective currents distinct from those of CFTR [45]. Several lines of evidence argued that cholapods function as mobile carriers rather than self-assembled channels: cholapod-mediated anion transport was dependent on transporter concentration, membrane fluidity and membrane thickness [45,46].

Using the common design motif of axially-directed hydrogen-bond donors in 1,5-relationships, the Anthony P. Davis group have developed other anionophores, including decalins [47,48] and cyclohexanes [49]. To evaluate the biological activity of anionophores, a fluorescence-based protocol was developed using halide-sensitive yellow fluorescent protein (YFP)-H148Q/I152L [50,51], which has been used with great success to identify small molecule CFTR modulators. Among the anionophores tested by Li et al. [50], a bis-(*p*-nitrophenyl)ureidodecalin was shown to be highly active using both the fluorescence-based protocol and the Ussing chamber technique (Figure 3). This anionophore was deliverable to cell membranes, potent at micromolar concentrations and its activity was long-lasting [50]. Moreover, when tested on three different epithelial cell lines, the bis-(*p*-nitrophenyl)ureidodecalin was without cytotoxic effects [50]. One possible explanation for the notable biological activity of the bis-(*p*-nitrophenyl)ureidodecalin is that among all the anionophores tested by Li et al. [50], it most closely satisfied Lipinski's rule of five for drug-like molecules [52].

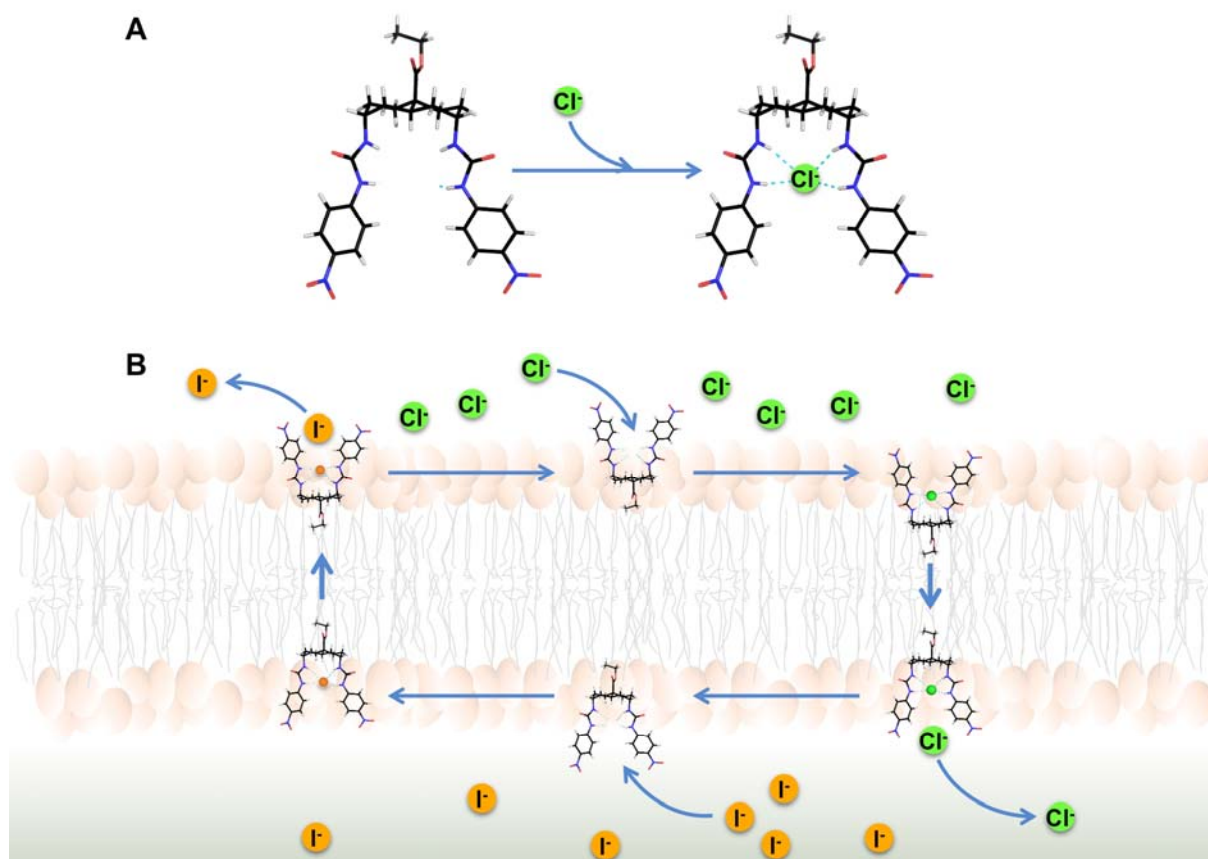


Figure 3: Transmembrane anion transport by anionophores. A, the crystal structure of the anionophore bis-(*p*-nitrophenyl)ureidodecalin in the absence and presence of Cl⁻. Dashed lines denote hydrogen bonds that bind the Cl⁻ ion. B, schematic of transmembrane anion transport by anionophores. The bis-(*p*-nitrophenyl)ureidodecalin anionophore is shown transporting I⁻ across the membrane in exchange for Cl⁻, which occurs when anionophores mediate the uptake of I⁻ by YFP-expressing Fischer rat thyroid cells leading to fluorescence quenching.

The identification of an anionophore with promising biological activity argues that anionophores merit further investigation as a mutation-independent therapy for CF. However, additional understanding of anion transport by anionophores is required, including structure-activity relationships, mechanism of action and cell biology. Like valinomycin, which shields transported K⁺ within a lipophilic shell, anionophores with encapsulated Cl⁻-binding sites achieve greater Cl⁻-selectivity [53]. Thus, optimisation of the chemical structure of anionophores will likely lead to anionophores most suitable for CFTR bypass therapy.

CONCLUSIONS

The widespread impact of CF mutations on CFTR processing argues that efficacious small molecule CFTR correctors are essential to develop disease-modifying therapies for CF patients. The data suggest that combinations of pharmacological chaperones that act directly on mutant CFTR will be required to achieve optimal rescue of the plasma membrane expression and stability of CF mutants [15,16]. Of note, the first of these CFTR corrector combination therapies recently gave positive results in phase 1 and phase 2 clinical trials (<http://investors.vrtx.com/releasedetail.cfm?ReleaseID=1033559>). While it is not known whether combination therapy begun early and used continuously will prevent disease symptoms developing over time, the data emerging from clinical trials are very encouraging. Mutant CFTR rescue using CFTR correctors might be complemented with proteostasis regulators, which target molecular steps in CFTR processing, delivery to and stability at the plasma membrane. The use of these agents might remove bottlenecks that obstruct the rescue of mutant CFTR. However, proteostasis regulators might have untoward effects because they target key steps in the biosynthesis, traffic and stability of other proteins besides CFTR. Future studies should address this possibility. Mutation-independent therapies will likely be required for individuals with certain rare CF mutations resistant to rescue with small molecule CFTR modulators and proteostasis regulators. Among the mutation-independent therapeutic strategies under consideration, anionophores might bypass CFTR dysfunction by shuttling Cl^- across the apical membrane of CF airway epithelia. Lessons from clinical trials of gene therapy [54] will need to be followed to optimise the delivery of anionophores to CF airway epithelia, while their activity might be controlled by modulation of basolateral membrane K^+ channels [55]. Beyond the respiratory airways, innovative drug delivery strategies will be required to specifically target anionophores to other epithelial tissues affected by CF without causing untoward effects. Thus, the challenge now is to translate laboratory discoveries into disease-modifying therapies for all individuals living with CF.

ACKNOWLEDGEMENTS

We thank our laboratory colleagues and collaborators for stimulating discussions. Work in the authors' laboratories discussed in this manuscript was supported by the Engineering and Physical Sciences Research Council [grant nos. EP/F03623X/1 and EP/J00961X/1] (HL and DNS), the Italian Cystic Fibrosis Foundation [FFC no. 5/2012 and FFC no. 2/2015] (NP), and the project teams working as part of the active collaboration between Abbvie, Inc. [www.abbvie.com] and Galapagos [www.glpg.com] (AKS). This review uses published research only. No new data were collected or generated in its preparation.

CONFLICT OF INTEREST STATEMENT

EP, NP, HL and DNS have no conflicts of interest to declare. AKS is an Abbvie employee.

AUTHOR CONTRIBUTIONS

All authors drafted the review manuscript or revised it critically for important intellectual content. All authors approved the final version of the review manuscript.

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